Remarkable in the study is that the exceptionally comprehensive analysis facilitated extraction of information about interconnectivity of dysregulated genes and identification of novel key regulatory node genes that might be important for aortic calcification. Thus, the work by Theodoris et al. represents a significant step forward in shedding light on the mechanism of aortic valve calcification, the epigenome and transcriptome in a human disease model of which can be altered by dose-reduction of a transcription factor, illustrating possible pathways for therapeutic intervention.

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Converting a Problem into an Opportunity: mtDNA Heteroplasmy Shift

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The transmission of mitochondrial DNA (mtDNA) disease from a mother with a heteroplasmic mtDNA mutation to her children is unpredictable. In a recent issue of *Cell*, Reddy et al. (2015) present the potential for mitochondrial-targeted nucleases to remove mutated mtDNA through the induction of heteroplasmy shift in oocytes and zygotes.

Mitochondria are the centerpiece of cellular metabolic machinery and contain multiple copies of a small genome, the mtDNA. Because an individual cell contains hundreds of mitochondria, each with its own varying genome, there is a substantial amount of mitochondrial genomic diversity, and this phenomenon is referred to as mtDNA heteroplasmy. Diseases associated with mtDNA mutations are unexpectedly common and represent a broad range of deteriorating conditions. The estimated incidence of mtDNA disease in adults is 1 in 5,000, but low levels of pathogenic mutations have been detected in 1 out of 200 births. Pathogenic mutations provoke symptoms only when mutant mtDNA is above a specific threshold, and every new mtDNA mutation creates a heteroplasmic mixture. Thus, a shift, or change in percentage of mutant alleles through replicative segregation, in mtDNA heteroplasmy is responsible for the pathogenicity of mitochondrial diseases. The mechanism by which this segregation occurs in either somatic or germ cells remains unknown (Wai et al., 2008). Importantly, mutated copies of mtDNA in the oocyte are transmitted to the embryo, but transmission of mtDNA disease from a mother with a heteroplasmic mtDNA mutation to her children is unpredictable. Preimplantation genetic diagnosis of embryos in affected mothers can reduce, but not eliminate, mitochondrial disease due to uncontrolled heteroplasmy. Therefore, affected mothers have no real choices for having healthy children except to play a form of reproductive "roulette," where they are left to choose between risking the possibility of their child having disabilities and a shortened life or terminating their pregnancy.

In a recent issue of *Cell*, Reddy and colleagues address this issue by exploiting mtDNA heteroplasmy as a therapeutic opportunity rather than an insurmountable problem (Reddy et al., 2015). Also, they take advantage of the poor capacity of the mitochondria to repair damaged mtDNA. To this end they use mitochondrial-targeted nucleases to remove mutated mtDNA in oocytes and zygotes

inducing specific damage in mutated mtDNA. Their approach is an attractive strategy for future therapy of mtDNA disorders wherein pathogenic conditions can be prevented without completely removing mutant alleles by simply dropping their levels below a specific threshold.

As a proof of concept, the authors first used the NZB/BALB heteroplasmic mice, which contain two mtDNA haplotypes, BALB and NZB, and selectively prevented their germline transmission using either mitochondria targeted restriction endonucleases or TALENs designed to remove BALB mtDNA (mito-ApaL1 nuclease) or NZB mtDNA (NZB-TALEN). Next they worked with two known human mtDNA diseases: Leber hereditary optic neuropathy (LHON), characterized by bilateral, painless, subacute visual failure that develops during young adult life, and NARP (neurogenic muscle weakness, ataxia, and retinitis pigmentosa), characterized by proximal neurogenic muscle weakness with sensory neuropathy, ataxia, and pigmentary retinopathy. Reddy et al. designed mito-TALENs targeted for the selective elimination of human mitochondrias harboring mutations of LHOND m.14459G>A and NARP m.9176T>C in human oocytes. The use of designed restriction nucleases and heterodimeric zinc finger nucleases for the induction of heteroplasmy shift has been previously demonstrated in human somatic cells (Bacman et al., 2013; Gammage et al., 2014). In the case of mtZFNS, Gammage et al. achieved a reduction in mutant mtDNA haplotype load following repopulation of wild-type mtDNA and restored mitochondrial respiratory function by shifting to the healthy mitochondrial population.

Targeting genome-editing tools such as ZFNs, TALENs, and CRISPR to the mitochondria has outstanding potential to reduce pathogenic mtDNA mutations; however, several limitations must be overcome prior to their use in the clinic. In this paper, Reddy and colleagues have resolved one of the key challenges by using mRNA injected into the oocyte to express LHOND and NARP mito-TALEN. Mouse oocytes were fused to fibroblasts and expression of mito-TALENs removed mutant mtDNA, generating a heteroplasmy shift. The use of RNA circumvents the disadvantages of exogenous DNA administration, such as the "three-genome baby" issue (Callaway, 2014). In addition, RNA is easier to handle compared to more sophisticated spindle transfer techniques.

However, it is important to acknowledge that genetic mis-targeting poses several potential problems: many of the potential targets require discrimination from indistinguishable regions in non-target mtDNA using only one base. Additionally, achieving high efficiency of importing endonucleases specifically into mitochondria is critical to avoid off-target effects in the nuclear genome, particularly for ZFNs, which have an intrinsic tropism for the nucleus (Bacman et al., 2014). Moreover, reducing mtDNA in the early embryo may be problematic, as no mtDNA replication is believed to occur between fertilization and post-implantation stages (Pikó and Taylor, 1987). The total amount of mtDNA is split among cells during embryonic division; thus, by day 5 of development, each embryonic cell likely contains few copies of mtDNA. Nevertheless, some data indicate that partial/mutant mtDNA depletion presumably would not impact embryo viability and implantation potential. Indeed, *Tfam* knockout mice, despite fewer mitochondria in oocytes, exhibit normal fertilization and early development (Wai et al., 2010). Further, mitochondria in mammalian oocytes have few cristae, which suggests that they have poor oxidative phosphorylation capacity. Combined, these observations suggest that the embryo is largely dependent on energy accumulation during oocyte maturation; thus, a reduction in mtDNA at early embryonic stages should not greatly affect the development potential.

The potential to use a heteroplasmatic shift approach is further supported by the observation that mitochondrial dysfunction is often associated with substantial mitochondrial hyperproliferation. Consequently, partial mtDNA depletion may stimulate an increase in non-pathogenic mtDNA copy number in early embryos. This has been demonstrated recently by Monnot et al. (2013), who show that the m.3243A>G MELAS mutation produces a gradual increase in mtDNA copy number from the germinal vesicle oocyte to the blastocyst stage. Thus, we can speculate that partial depletion of mutated mtDNA will stimulate mitochondrial biogenesis, giving non-targeted mitochondria an advantage in displacing the mutant population.

The heteroplasmic shift approach using mitochondrial target nucleases is promising for reducing the transmission of pathogenic mtDNA. Future improvements of this technology will involve the use of purified nuclease protein or even a combination of RNA and protein at the meiosis II oocyte stage. In order to make specific nuclease design for each mutation affordable, we will require better molecular and bioinformatics tools that will be available in the near future.

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